"Effectiveness of the methanolic extract from *Euryale ferox* seeds in regulating cellular glutathione levels in Zebrafish exposed to tobacco-specific nitrosamine."

Synopsis

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Introduction

Since time immemorial, it has been asserted that the remedies for various diseases can be found in nature. As our lifestyles become increasingly complex, the incidence of diseases is on the rise, necessitating reliance on pharmaceuticals that are often accompanied by undesirable side effects. Nevertheless, natural alternatives to chemically synthesized medicines have been proven to be a blessing due to their ready availability, effectiveness, cost-effectiveness, and minimal side effects.

Tobacco consumption is a lifestyle habit known to be associated with carcinogenesis in various organs, including the lungs, throat, buccal cavity, esophagus, stomach, and even the liver. Despite the availability of chemotherapeutic agents in the market, ongoing research is being undertaken to seek better alternatives that can minimize the harmful effects of chemicals and enhance the quality of life post-treatment.

Within the realm of natural products, efficacy as remedies for cancer has been demonstrated by the active compounds or metabolites found in curcumin, ginger, garlic, ginkgo, ginseng, and other such agents (Ramana et al., 2014). Currently, there is a growing emphasis on alternative medicines, Ayurvedic treatments, and naturopathy as solutions to combat diseases. Amidst the increasing popularity of plant-based remedies, a medicinal plant often overlooked is abundantly grown in the ponds of the Mithilanchal region of Bihar – Euryale ferox seeds, also known as foxnuts.

Euryale ferox Salisb. (Nymphaeaceae), commonly referred to as Makhana, Phool Makhana, Gorgon Nut, or Fox Nut in India, stands as the sole member of the Euryale genus. Abundant in the Bihar regions of Mithila, Darbhanga, and Madhubani, this revered dry fruit holds a significant place in Ayurvedic medicinal practices. Its historical usage spans the treatment of renal disorders, chronic diarrhea, excessive

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leucorrhea, and hepatic dysfunction. Renowned for its bioactive compounds, it serves as an antioxidant, antimicrobial, antiischemic, antidiabetic, immunomodulatory, anti-melanogenic, and anti-cytotoxic agent.

It is widely acknowledged that tobacco is considered a potent carcinogen, and its classification as a Group 1 carcinogen by the IARC (IARC Monographs on the Identification of Carcinogenic Hazards to Humans, n.d.) is supported by mounting evidence. The carcinogenic process induced by tobacco involves the generation of Reactive Oxygen Species (ROS), the formation of DNA-adducts, the incapacity of Cytochrome P450 and related molecules for detoxification, base substitution, altered expression of specific genes, and impaired proteins (Gelboin, 2012; Hecht, 2003; Pfeifer et al., 2002). Depletion of Glutathione emerges as a crucial factor amplifying the adverse effects of tobacco-related carcinogens, particularly Nitrosamines. The significance of glutathione availability in close proximity to the toxin for efficient elimination was highlighted in a recent study (Yadav et al., 2020).

Ongoing research is exploring various naturally occurring substances, though Euryale ferox seeds (foxnuts) remain relatively unexplored as potential anti-cancer agents. Excellent antioxidant properties have been demonstrated by these foxnuts (Lee et al., 2002a), suggesting potential implications for a protective role against cancer.

Darbhanga, renowned for its wetlands prone to frequent flooding during the monsoon, optimally utilizes these conditions for cultivating foxnuts. This practice significantly contributes to the economic growth of the city. Despite foxnut being recognized as a superfood, its potential protective or curative properties against cancer are yet to be fully understood.

Ample initial knowledge concerning the carcinogenic consequences associated with tobacco exists, leading to cellular damage through various compounds such as aromatic hydrocarbons and nitrosamines, which are extensively documented. Current therapies for tobacco-induced cancer predominantly revolve around rectifying cellular damage and eliminating affected cells or tissues, with limited emphasis on detoxifying tobacco-related toxins. A substantial intracellular pool of reduced Glutathione (GSH) is necessitated by the collaboration of Glutathione S-transferase with other factors to bind with toxins and expel them from the system. Thus, the intracellular reservoir of Glutathione can significantly influence cancer prognosis.

While the antioxidant qualities of Euryale ferox seeds are acknowledged, the direct correlation remains ambiguous. Among diverse extracts and fractions, the ethyl acetate fraction of the methanolic extract (EFSM) emerges as the most potent antioxidant carrier, as suggested by research. Although multiple factors may contribute to its antioxidant nature, the adjustment of the reduced glutathione (GSH) to oxidized glutathione (GSSG) ratio and the maintenance of the Glutathione pool cannot be dismissed.

Literature Review

In a notable recognition of its distinctive qualities and regional importance, the prestigious Geographical Indication (GI) tag has been bestowed upon Makhana from the Mithila region. The exceptional characteristics and traditional practices associated with Makhana cultivation in Mithila are highlighted by this acknowledgment.

A comprehensive study on the potential impact of the ethanolic extract of Euryale ferox salisb. (EFx) on hepatic antioxidant enzyme activity, glycemic control, lipid profile, and the histopathology of the pancreas, liver, and kidney in streptozotocin (STZ)-induced

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diabetic Wistar rats was conducted by Ahmed et al. (Ahmed et al., 2015). Another promising study by Vikram et al. revealed that the diverse array of bioactive constituents present in Euryale ferox demonstrates various activities, including antioxidant, antidiabetic, anti-hyperlipidemic, hepatoprotective, anti-cytotoxic, cardioprotective, and antidepressant effects, among others. Given these attributes, Euryale ferox could be served as a potential supplement for Covid-19 patients (Vikram & Mishra, 2021). Despite these findings, further research on Euryale ferox is still needed, especially considering its abundant cultivation in the wetlands of the northern plains of India.

The historical use of Euryale ferox in Eastern medicine has been extensively recorded, yet the exact cellular mechanisms underpinning its therapeutic effects remain relatively unexplored. To address this gap, an examination into the antioxidant properties of extracts and fractions derived from Euryale ferox was conducted by researchers.

Euryale ferox seeds (EFS) have been enjoying a revered status in Chinese culture for more than two millennia due to their exceptional nutritional composition. Enriched with essential elements such as polysaccharides, polyphenols, sesquineolignans, tocopherols, cyclic dipeptides, glucosylsterols, cerebrosides, and triterpenoids, a diverse range of medicinal benefits is offered by these seeds. These include antioxidant properties, regulation of blood sugar levels, cardiovascular protection, antibacterial efficacy, anticancer potential, antidepressant effects, and liver safeguarding.

Upon investigation, it was noted that ethyl acetate fractions, along with butanol fractions and total extracts from Euryale ferox, progressively enhanced the activities of crucial antioxidant enzymes—superoxide dismutase, catalase, and glutathione peroxidase—in V79-4 cells. Notably, glutathione peroxidase exhibited the most

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significant increase in activity, indicating substantial antioxidant capabilities in Euryale ferox. Additionally, specific constituents found in the ethyl acetate and butanol fractions appear to play a crucial role in driving these notable antioxidant effects (Lee et al., 2002b). Another study on the ethyl acetate fraction of Euryale ferox seed extract (Efse-EA) demonstrated its impact on melanin production in melan-a cells. Efse-EA exhibited a strong ability to inhibit tyrosinase, leading to a reduction in melanin production and related proteins. This suggests that Efse-EA may impede melanin formation by affecting how tyrosinase breaks down in lysosomes, hinting at its potential use as a skin-lightening agent in cosmetic applications (Baek et al., 2015).

Concerning the conjugation of glutathione catalyzed by Glutathione-S-transferases (GSTs), it has been well-characterized in zebrafish (Danio rerio), recognizing their pivotal role in the second phase of cellular detoxification. Zebrafish, widely utilized as a vertebrate model in environmental research, was chosen due to its relevance in studying these enzymes (Glisic et al., 2015).

The effects of tobacco-nitrosamines have been documented in zebrafish embryos exposed to various concentrations of nitrosamine at 6 hours post-fertilization (hpf). Results indicated negative impacts on the hatching rate, heartbeat, body length, and spontaneous movement due to nitrosamine exposure. Deformities, including notochord malformation, pericardium edema, embryonic membrane turbidity, tail hypoplasia, yolk sac deformity, and growth retardation, were observed during the exposure period. Furthermore, there was a significant increase in the production of reactive oxygen species (ROS) following nitrosamine treatment, accompanied by observed changes in the expression levels of genes associated with oxidative stress (Huang et al., 2018).

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Objectives

Based on existing evidence and our recent discoveries, the objectives outlined in the synopsis are as follows:

1. Evaluate the harm induced by tobacco-nitrosamine in the liver of zebrafish.
2. Analyze the alteration in glutathione levels between nitrosamine-treated and untreated zebrafish.
3. Observe the variation in glutathione levels in nitrosamine-treated zebrafish before and after the administration of *Euryale ferox* seed methanolic extract (EFSM).

Plan of work

In the initial phase, the necessary chemicals, reagents, kits, glassware/plasticware, and equipment will be acquired by us. Zebrafish procurement will be conducted through authorized agencies, and the acquired fish will be housed in tanks for approximately 6 weeks to undergo acclimatization. A distinct group of zebrafish will be segregated for breeding purposes, facilitating embryo collection for subsequent studies.

Moving on to the second phase of the study, acclimatized zebrafish (10 in each concentration group, including the untreated group) will be subjected to intraperitoneal injections with 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) dissolved in PBS. The injections will be administered at varying concentrations, ranging from low to high, in separate tanks every alternate day for a duration of 21 days. The treatment dosage will be meticulously standardized, and the experiment will be conducted at least three times to ensure data reliability. Fish mortality will be recorded in correlation with the injected amount of NNK, allowing the calculation of the 50% mortality rate.

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In the subsequent phase, following the determination of the LD50, the sublethal dose will be identified to ensure optimal viability of Zebrafish. A standardized protocol for estimating intracellular Glutathione (GSH) levels will be developed, and the Methanolic extract of Euryale ferox seeds (EFSM) will be prepared in accordance with the methodology outlined.

Moving to the fourth phase, zebrafish previously treated with NNK will receive intraperitoneal injections of EFSM proportional to their body weight (approximately 5 individuals). Biochemical parameters will be examined and compared to those of NNK-treated zebrafish without EFSM supplementation to assess the potential curative effects of EFSM.

In the final phase, statistical computations will be conducted, and the gathered data will be compiled for scientific reporting.

Expected outcome

This study represents the first attempt to address a local health concern, tobacco consumption, by exploring a locally available remedy. Given the reported antioxidant properties of Euryale ferox seeds, it is anticipated that EFSM can mitigate the damages caused by tobacco. The central focus of this study is to investigate whether this protective effect is linked to the modulation of intracellular GSH for effective detoxification.

To elaborate, an abrupt decline in glutathione levels within cells following tobacco-nitrosamine treatment is anticipated. This decrease would signify the unavailability of GSH for Glutathione S-transferase, hindering efficient detoxification and providing insights into the potential carcinogenesis in affected tissues. With the administration of

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EFSM, it is expected to observe a sufficient GSH pool, facilitating the efficient detoxification of nitrosamines.

Successful achievement of our objectives would open avenues for future research in several areas. Firstly, the fraction responsible for effective detoxification (such as Ethyl acetate, Butanol, Hexane, etc.) would be isolated. Secondly, any novel compounds could be identified, laying the groundwork for drug design and production. Thirdly, this study could serve as a guiding beacon for further anticancer research using Euryale ferox seeds. Fourthly, Euryale ferox might warrant consideration for additional research on cancers caused by different carcinogens. Lastly, the potential stimulation of the local economy by encouraging the establishment of industries for foxnut production, contributing to the sustainable development of the region, is highlighted by this research..

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Conclusion

Renowned for its nutritional richness, Euryale ferox seeds are acknowledged as a superfood with proven benefits such as diabetes prevention, blood pressure control, fertility enhancement, weight maintenance, and overall health promotion. Despite recent research highlighting its potential as an antioxidant, a conclusive link between these seeds and their role in combatting cancer is yet to be established. This study aims to investigate whether the methanolic extract of Euryale ferox seeds (EFSM) can offer protection or a remedy against the damages induced by tobacco-nitrosamine NNK. The focus is on understanding if this protective effect is achieved through the modulation of cellular glutathione status in zebrafish.

Leveraging the close genetic resemblance to humans and ease of maintenance, Zebrafish will be utilized for a comprehensive range of experiments. This will include determining the LD50 for NNK treatment and estimating glutathione levels in NNK+EFSM treated fish. The findings from this study will serve as a foundation for future research endeavors, whether in the realm of drug discovery or elucidating the molecular mechanisms underlying the activity of Euryale ferox within cellular contexts.

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